

NEUROTROPHIC FACTOR RECEPTOR5 **Cross Reference to Related Applications**

This Application claims priority under 35 U.S.C § 371 to PCT/EP00/04918 filed May 26, 2000 which claims priority from Great Britain Patent Application No. 9915200.1 filed June 29, 1999 and entitled "Neurotrophic Factor Receptor."

10 **Background of the Invention**

15 The present invention is concerned with cloning and expression of a novel mammalian receptor protein, designated herein GFR $\alpha$ -4 and in particular with an isolated nucleic acid sequence encoding the GFR $\alpha$ -4 protein, an expression vector comprising said nucleic acid sequence, a host cell transformed or transfected with said vector, isolated GFR $\alpha$ 4 protein, compounds which act as agonists or antagonists in relation to GFR $\alpha$ -4 and methods of identifying them, together with pharmaceutical compositions comprising the isolated nucleic acid, the receptor protein or said agonist or 20 antagonist.

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30 Neurotrophic growth factors are involved in neuronal differentiation, development and maintenance. These proteins can prevent degeneration and promote survival of different types of neuronal cells and are thus potential therapeutic agents for neurodegenerative diseases. Glial cell-line derived neurotrophic factor (GDNF) was the first member of a growing subfamily of 35 neurotrophic factors structurally distinct from the neurotrophins. GDNF is a distantly related member of the transforming growth factor  $\beta$  (TGF- $\beta$ ) superfamily of growth factors, characterized by a specific pattern of seven highly conserved cysteine residues within the 40 amino acid sequence (Kingsley, 1994). GDNF was

originally purified using an assay based on its ability to maintain the survival and function of embryonic ventral midbrain dopaminergic neurons *in vitro* (Lin et al., 1993). Other neuronal cell types in the central (CNS) or peripheral nervous systems (PNS) have been shown to be responsive to the survival effects of GDNF (Henderson et al., 1994, Buj-Bello et al., 1995, Mount et al., 1995, Oppenheim et al., 1995). GDNF is produced by cells in an inactive proform, which is cleaved specifically at a RXXR recognition site to produce active GDNF (Lin et al., 1993). In view of its effects on dopaminergic neurons, clinical trials have evaluated GDNF as a possible treatment for Parkinson's disease, a common neurodegenerative disorder characterized by the loss of a high percentage (up to 70 %) of dopaminergic cells in the substantia nigra of the brain. Exogenous administration of GDNF has potent protective effects in animal models of Parkinson's disease (Henderson et al., 1994, Beck et al., 1995, Tomac et al., 1995, Yan et al., 1995, Gash et al., 1996, Choi-Lundberg et al., 1997, Bilang-Bleuel et al., 1997, Mandel et al., 1997).

Recently, three new members of the GDNF family of neurotrophic factors have been discovered. Neurturin (NTN) was purified from conditioned medium from Chinese hamster ovary (CHO) cells using an assay based on the ability to promote the survival of sympathetic neurons in culture (Kotzbauer et al., 1996). The mature neurturin protein is 57% similar to mature GDNF. Persephin (PSP) was discovered by degenerate primer PCR using genomic DNA. The mature protein, like mature GDNF, promotes the survival of ventral midbrain dopaminergic neurons and of motor neurons in culture (Milbrandt et al., 1998). The similarity of the mature persephin protein with mature GDNF and neurturin is ≈ 50 %. Very recently, a fourth member has been cloned using genomic DNA information in the public EMBL database and has been named Enovin (EVN) (Masure et

al., 1999) or Artemin (ARTN) (Baloh et al., 1998b). This factor is  $\pm$  57 % similar to NTN and PSP and acts primarily on peripheral neurons.

5 All four GDNF family members require a heterodimeric receptor complex in order to carry out downstream intracellular signal transduction. GDNF binds to the GDNF family receptor alpha 1 (GFR $\alpha$ -1; also termed GDNFR $\alpha$ , RETL1 or TrnR1; GFR $\alpha$  Nomenclature Committee, 1997) subunit, a glycosyl phosphatidyl inositol (GPI)-10 anchored membrane protein (Jing et al., 1996, Treanor et al., 1996, Sanicola et al., 1997). The GDNF/GFR $\alpha$ -1 complex subsequently binds to and activates the cRET proto-oncogene, a membrane bound tyrosine kinase (Durbec et al., 1996, Trupp et al., 1996), resulting in phosphorylation of tyrosine residues in cRET and 15 subsequent activation of downstream signal transduction pathways (Worby et al., 1996). GFR $\alpha$ -2 (also termed RETL2, NTN $\alpha$ , GDNFR $\beta$  or TrnR2), which 20 is similar to GFR $\alpha$ -1, has been identified by a number of different groups (Baloh et al., 1997, Sanicola et al., 1997, Klein et al., 1997, Buj-Bello et al., 1997, Suvanto et al., 1997). The human GFR $\alpha$ -1 and GFR $\alpha$ -2 25 receptor subunits are 49% identical and 63% similar by protein sequence with 30 of the 31 cysteine residues conserved. Both receptors contain a hydrophobic domain at their carboxy-termini involved in GPI anchoring to the membrane. GFR $\alpha$ -1 and GFR $\alpha$ -2 are widely expressed 30 in almost all tissues and expression may be developmentally regulated (Sanicola et al., 1997, Widenfalk et al., 1997).

35 GFR $\alpha$ -1 is the preferred receptor for GDNF, whereas GFR $\alpha$ -2 preferentially binds neurturin (Jing et al., 1996, Treanor et al., 1996, Klein et al., 1997). It is also clear, however, that there is some cross-talk 40 between these growth factors and receptors as GDNF can bind to GFR $\alpha$ -2 in the presence of cRET (Sanicola et al., 1997) and neurturin can bind to GFR $\alpha$ -1 with low affinity (Klein et al., 1997). GDNF and neurturin are

thus part of a neurotrophic signalling system whereby different ligand-binding subunits (GFR $\alpha$ -1 and GFR $\alpha$ -2) can interact with the same tyrosine kinase subunit (cRET).

Recently, a third member of the GFR $\alpha$  family of coreceptors, GFR $\alpha$ -3, has been described (Jing et al., 1997, Masure et al., 1998, Worby et al., 1998, Naveilhan et al., 1998, Baloh et al., 1998a). This receptor's amino acid sequence is 35% identical to both GFR $\alpha$ -1 and GFR $\alpha$ -2. GFR $\alpha$ -3 is not expressed in the developing or adult CNS, but is highly expressed in several developing and adult sensory and sympathetic ganglia of the PNS (Widenfalk et al., 1998, Naveilhan et al., 1998, Baloh et al., 1998a). GFR $\alpha$ -3 has been shown to be the preferred coreceptor for Enovin/artemin and also signals via cRET (Masure et al., 1999, Baloh et al., 1998b). Crosstalk between EVN/ARTN and GFR $\alpha$ -1 seems also possible, at least *in vitro*.

A fourth member of the GFR $\alpha$  family has been identified in chicken (Thompson et al., 1998) and has been shown to mediate signalling of persephin via cRET (Enokido et al., 1998). A functional mammalian homologue encoding a mammalian persephin receptor has yet to be discovered.

The present inventors have surprisingly identified a further novel mammalian receptor of the GDNF family designated herein as GFR $\alpha$ -4. The DNA sequence has been cloned and a number of splice variants encoding the receptor have also been identified.

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#### Summary of the Invention

Accordingly, there is provided by the present invention an isolated or substantially pure form of a nucleic acid encoding a mammalian GDNF family receptor  $\alpha$ -4 designated GFR $\alpha$ -4. The nucleic acid molecule is

preferably from rat, mouse or human. Preferably, the receptor encoded by said nucleic acid molecule comprises the amino acid sequence illustrated in Sequence ID No's. 8 or 9 or encoding a functional equivalent, derivative or bioprecursor of said receptor.

Although initially, in view of the fact that only 4 members of the GFR $\alpha$ s were known, the new receptor has previously been called GFR $\alpha$ -5. However, it has now 10 termed  $\alpha$ -4 in order to comply with the existing nomenclature for GFR $\alpha$  family members and to indicate that the GFR $\alpha$  receptor of the present invention is the mammalian orthologue of the chicken GFR $\alpha$ -4 receptor.

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#### **Brief Description of the Figures**

20 The present invention may be more clearly understood from the following exemplary embodiment with reference to the accompanying figures wherein;

25 Figure 1: is an illustration of the Structure of the rat GFR $\alpha$ -4 gene. The top line shows a scale in bp. The line below shows the genomic structure of the rat GFR $\alpha$ -4 gene. Exons are represented by boxes and numbered, intron sequences are depicted as lines. The sizes (in bp) of introns and exons are indicated above the diagram. The translation start codon is indicated by an arrow and the stop codon by an asterisk. The cDNA sequences of variants A and B obtained by 30 splicing out the intron sequences is shown below the genomic sequence. Alternative splicing of intron 5 results in an earlier stop codon in splice variant B. The predicted protein sequences of variants A and B are shown at the bottom. The predicted signal peptide, 35 a putative N-glycosylation site and a hydrophobic COOH-terminal region preceded by one or two possible sites for GPI-cleavage (in variant A only) are indicated on the diagrams.

Figure 2: is an alignment of the predicted protein sequences of splice variants A and B of rat GFR $\alpha$ -4. The sequences of rat GFR $\alpha$ -4 splice variants A and B were aligned using the ClustalW alignment program (EMBL, Heidelberg, Germany). Amino acid residues conserved between the 2 variants are included in the black areas. Amino acid residues are numbered to the right. The dashes indicate gaps introduced into the sequence to optimize the alignment.

Figure 3: is an alignment of the predicted protein sequences of GFR $\alpha$  family members. The sequence of rat GFR $\alpha$ -4 variants A and B, rat GFR $\alpha$ -1 (EMBL acc. no. U59486), rat GFR $\alpha$ -2 (EMBL acc. no. AF003825), mouse GFR $\alpha$ -3 (EMBL acc. no. AB008833) and chicken GFR $\alpha$ -4 (EMBL acc. no. AF045162) were aligned using the ClustalW alignment program (EMBL, Heidelberg, Germany). Amino acid residues conserved between all 6 proteins are included in the black areas. Residues conserved between 4 or 5 of the sequences are shaded in grey. Cysteine residues conserved between all six GFR $\alpha$ 's are indicated with an asterisk above the sequence. Amino acid residues are numbered to the right. The dashes indicate gaps introduced into the sequence to optimize the alignment.

Figure 4: Northern blot analysis of rodent GFR $\alpha$ -4 mRNA expression. The expression of rat and mouse GFR $\alpha$ -4 mRNA in different tissues was assessed using a probe corresponding to the coding sequence of rat GFR $\alpha$ -4 to analyze blots of poly(A)-rich RNA. (A) Rat Multiple Tissue Northern (MTN) blot; (B) Mouse MTN blot and (C) Mouse Embryo MTN blot. Apparent sizes are indicated (in kilobase pairs) by horizontal lines to the left of each panel.

Figure 5. The rat GFR $\alpha$ -4 gene is localized on chromosome 3q36. A mixture of two rat GFR $\alpha$ -4 probes was used for FISH analysis. (A) Double-spot FISH signals on the middle-distal part of rat chromosome 3

(arrows). (B) Position of the GFR $\alpha$ -4 gene locus on rat chromosome 3q36.

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### Detailed Description of the Invention

Thus, the present invention relates to a nucleic acid molecule encoding a mammalian GDNF family receptor  $\alpha$ -4 (GFR $\alpha$ -4) or an immunologically and/or biologically active fragment thereof, which comprises a nucleotide sequence selected from the group consisting of

10 (a) nucleotide sequences encoding the polypeptide comprising the amino acid sequence depicted in SEQ ID NO: 8 or 9;

15 (b) nucleotide sequences comprising the coding sequence as depicted in SEQ ID NO: 5 or 6;

20 (c) nucleotide sequences encoding a polypeptide derived from the polypeptide encoded by a nucleotide sequence of (a) or (b) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence encoded by the nucleotide sequence of (a) or (b);

25 (d) nucleotide sequences the complementary strand of which hybridizes with a nucleotide sequence of any one of (a) to (c);

30 (e) nucleotide sequences encoding a polypeptide the amino acid sequence of which has an identity of 30% or more to the amino acid sequence of the polypeptide encoded by a nucleotide sequence of any one of (a) to (d);

35 (f) nucleotide sequences encoding a polypeptide capable of binding persephin comprising a fragment or an epitope-bearing portion of a polypeptide encoded by a nucleotide sequence of any one of (a) to (e);

40 (g) nucleotide sequences comprising at least 15 consecutive nucleotides of a nucleotide sequence of any one of (a) to (f) and encoding a polypeptide capable of binding

persephin; and

(h) nucleotide sequences comprising a nucleotide sequence which is degenerated as a result of the genetic code to a nucleotide sequence of any of (a) to (g).

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Advantageously, the nucleic acid molecule according to the invention may be used for expression of said GFR $\alpha$ -4 protein in, for example, a host cell or the like, using an appropriate expression vector.

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Preferably, the nucleic acid molecule is a DNA molecule, and even more preferably a cDNA molecule having a sequence as illustrated in any of Sequence ID No's. 5 to 7 or the complement thereof.

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Alternatively, the nucleic acid molecule is capable of hybridising to the sequences of the invention under conditions of high stringency or to the complement thereof. Stringency of hybridisation as used herein refers to conditions under which polynucleic acids are stable. The stability of hybrids is reflected in the melting temperature ( $T_m$ ) of the hybrids.  $T_m$  can be approximated by the formula:

20

$$81.5^{\circ}\text{C} + 16.6(\log_{10}[\text{Na}^+]) + 0.41 (\% \text{G&C}) - 6001/1$$

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wherein 1 is the length of the hybrids in nucleotides.  $T_m$  decreases approximately by 1-1.5°C with every 1% decrease in sequence homology.

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The nucleic acid capable of hybridising to nucleic acid molecules according to the invention will generally be at least 70%, preferably at least 80 or 90% and more preferably at least 95% homologous to the nucleotide sequences according to the invention.

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Advantageously, the antisense molecule may be used as a probe or as a medicament or in a pharmaceutical composition together with a pharmaceutically acceptable carrier, diluent or excipient.

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According to a second aspect of the invention, there is provided a DNA expression vector comprising the DNA molecule according to the invention. This vector may, advantageously, be used to transform or transfect a host cell to achieve expression of GFR $\alpha$ -4 according to the invention. Preferably, the DNA is included in a plasmid, for subsequent transfection or transformation of the host cell.

An expression vector according to the invention includes a vector having a nucleic acid according to the invention operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxta position wherein the components described are in a relationship permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell to provide for expression of a polypeptide according to the invention. Thus, in a further aspect, the invention provides a process for preparing receptors according to the invention which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the receptors, and recovering the expressed receptors.

The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and

for transcription initiation in the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art.

Nucleic acid molecules according to the invention may be inserted into the vectors described in an antisense orientation in order to provide for the production of antisense RNA. Antisense RNA or other antisense nucleic acids may be produced by synthetic means.

In accordance with the present invention, a defined nucleic acid includes not only the identical nucleic acid but also any amino base variations including, in particular, substitutions in bases which result in a synonymous codon (a different codon specifying the same amino acid residue) due to the degenerate code in conservative amino acid substitutions. The term "nucleic acid sequence" also includes the complementary sequence to any single stranded sequence given regarding base variations.

The present invention also advantageously provides nucleic acid sequences of at least approximately 10 contiguous nucleotides of a nucleic acid according to the invention and preferably from 10 to 50 nucleotides. These sequences may, advantageously, be used as probes or primers to initiate replication, or the like. Such nucleic acid sequences may be produced according to techniques well known in the art, such as, by recombinant or synthetic means. They may also be used in diagnostic kits or the like for detecting the presence of a nucleic acid according to the invention. These tests generally comprise contacting the probe with the sample under hybridising conditions

and detecting for the presence of any duplex or triplex formation between the probe and any nucleic acid in the sample.

5 According to the present invention these probes may be anchored to a solid support. Preferably, they are present on an array so that multiple probes can simultaneously hybridize to a single biological sample. The probes can be spotted onto the array or  
10 synthesised *in situ* on the array. (See Lockhart *et al.*, Nature Biotechnology, vol. 14, December 1996 "Expression monitoring by hybridisation into high density oligonucleotide arrays"). A single array can contain more than 100, 500 or even 1,000 different probes in discrete locations.  
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20 The nucleic acid sequences, according to the invention may be produced using such recombinant or synthetic means, such as, for example, using PCR cloning mechanisms which generally involve making a pair of primers, which may be from approximately 10 to 50 nucleotides to a region of the gene which is desired to be cloned, bringing the primers into contact with mRNA, cDNA, or genomic DNA from a human cell,  
25 performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified region or fragment and recovering the amplified DNA. Generally, such techniques as defined herein are well known in  
30 the art, such as described in Sambrook *et al* (Molecular Cloning: a Laboratory Manual, 1989).

35 The nucleic acids or oligonucleotides according to the invention may carry a revealing label. Suitable labels include radioisotopes such as  $^{32}\text{P}$  or  $^{35}\text{S}$ , enzyme labels or other protein labels such as biotin or fluorescent markers. Such labels may be added to the nucleic acids or oligonucleotides of the invention and may be detected using known techniques *per se*.

The present invention also comprises within its scope proteins or polypeptides encoded by the nucleic acid molecules according to the invention or a functional equivalent, derivative or bioprecursor thereof.

5 Preferably, the protein comprises the amino acid sequence of Sequence ID No's. 8 and 9.

A "functional equivalent" as defined herein should be taken to mean a receptor that exhibits the same 10 properties and functionality associated with the GFR $\alpha$ -4 receptor according to the invention. A "derivative" should be taken to mean a polypeptide or protein in which certain amino acids may have been altered or deleted or replaced and which polypeptide 15 or protein retains biological activity of said GFR $\alpha$ -4 receptor and/or which can cross react with antibodies raised using a receptor according to the invention as the challenging antigen.

20 Encompassed within the scope of the invention are hybrid and modified forms of the GFR $\alpha$ -4 receptor according to the invention including fusion proteins and fragments. The hybrid and modified forms include, 25 for example, when certain amino acids have been subjected to some modification or replacement, such as for example, by point mutation and yet which results in a protein which possesses the same receptor specificity as the GFR $\alpha$ -4 receptor of the invention.

30 In this context it is understood that the terms biological activity, receptor specificity and functional receptor (fragment) preferably include the ability to bind persephin, preferably specifically and that the GFR $\alpha$ -4 of the invention has no or 35 substantially no binding activity on GDNF, NTN, and/or EVN/ART. The biological activity, receptor specificity and/or functionality, for example binding activity of the GFR $\alpha$ -4 of the invention, variants, derivatives and fragments thereof can be determined according methods 40 well known in the art, preferably as described in the

appended examples. Preferably, the  $K_d$  of the GFR $\alpha$ -4 of the invention for persephin is 1 to  $10 \times 10^{-9}$ , particularly preferred  $5.9 \pm 2.8 \times 10^{-9}$  when determined in accordance with the examples described below; see 5 the description to Table 6.

The protein according to the invention should be taken to include all possible amino acid variants encoded by the nucleic acid molecule according to the invention 10 including a polypeptide encoded by said molecule and having conservative amino acid changes. Proteins or polypeptides according to the invention further include variants of such sequences, including naturally occurring allelic variants which are 15 substantially homologous to said proteins or polypeptides. In this context, substantial homology is regarded as a sequence which has at least 70%, and preferably 80 or 90% amino acid homology with the proteins or polypeptides encoded by the nucleic acid 20 molecules according to the invention.

Substantial homology should be taken to mean that the nucleotide and amino acid sequences of the GFR $\alpha$ -4 of the invention display a certain degree of sequence 25 identity. Preferably they share an identity of at least 30 %, preferably 40 %, more preferably 50 %, still more preferably 60 %, most preferably 70%, and particularly an identity of at least 80 %, preferably more than 90 % and still more preferably more than 95 % is desired with respect to the nucleotide or amino 30 acid sequences depicted in Seq. ID Nos. 5 to 9, respectively. A preferred method for determining the best overall match between a query sequence (a sequence of the present invention) and a subject sequence, also referred to as a global sequence 35 alignment, can be determined using, for example, the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6 (1990), 237-245.) In a sequence alignment the query and subject 40 sequences are both DNA sequences. An RNA sequence can

be compared by converting U's to T's. The result of  
said global sequence alignment is in percent identity.  
Further programs that can be used in order to  
determine homology/identity are described below and in  
5 the examples. The sequences that are homologous to the  
sequences described above are, for example, variations  
of said sequences which represent modifications having  
the same biological function, in particular encoding  
proteins with the same or substantially the same  
10 receptor specificity, i.e. binding specificity. They  
may be naturally occurring variations, such as  
sequences from other mammals, or mutations. These  
mutations may occur naturally or may be obtained by  
mutagenesis techniques. The allelic variations may be  
15 naturally occurring allelic variants as well as  
synthetically produced or genetically engineered  
variants. In a preferred embodiment the sequences are  
derived from mouse, more preferable from human. These  
sequences can also be retrieved from existing  
20 databases with nucleotide sequences of yet unknown  
function. For example, a BLAST search on the EMBL  
database using the identified rat GFR $\alpha$ -4 sequences as  
query sequence yielded a genomic mouse sequence  
(accession no. AF155960) and a genomic human sequence  
25 (accession no. AC017113; containing contigs. derived  
from human genomic DNA) with parts almost identical  
with parts of the rat GFR $\alpha$ -4 sequence (exons 2, 3 and  
4). These nucleotides sequences are also encompassed  
in the present invention.

30 A further aspect of the invention comprises the host  
cell itself transformed with the DNA expression vector  
described herein, which host cell preferably comprises  
a eukaryotic cell, which may be for example, a  
35 mammalian cell, an insect cell or yeast cell or the  
like. In one embodiment the cell comprises a human  
embryonic kidney cell and preferably a cell of the  
HEK293 cell line. Alternatively, the cell may  
comprise NIH/3T3 mouse fibroblasts or Chinese hamster  
40 ovary (CHO) cells or COS-7 cells.

Further provided by the present invention is a transgenic cell, tissue or organism comprising a transgene capable of expressing GFR $\alpha$ -4 according to the invention, or of expressing a functional equivalent, derivative or bioprecursor of said receptor. The term "transgene capable of expression" as used herein means a suitable nucleic acid sequence which leads to expression of a human receptor having the same function and/or activity as GFR $\alpha$ -4. The transgene may include, for example, genomic nucleic acid isolated from rat cells or synthetic nucleic acid, including cDNA, integrated into the genome or in an extra chromosomal state. Preferably, the transgene comprises the nucleic acid sequence encoding GFR $\alpha$ -4 according to the invention or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene or cDNA encoding GFR $\alpha$ -4 receptor or a functional equivalent or bioprecursor of said GFR $\alpha$ -4 which fragment is capable of being expressed to produce a functional receptor protein. For example, the gene may comprise deletions of mutations but may still encode a functional receptor.

Further provided by the present invention is an isolated or purified GFR $\alpha$ -4 protein having the amino acid sequence illustrated in Sequence ID No. 2 or a functional fragment or bioprecursor of said receptor or alternatively a GFR $\alpha$ -4 protein expressed by the transgenic cell, tissue or organism according to the invention. Also provided by the invention are membrane preparations from cells expressing GFR $\alpha$ -4.

The present invention is further directed to inhibiting GFR $\alpha$ -4 *in vivo* by the use of antisense technology. Antisense technology can be used to control gene expression through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA.

For example, the 5' coding portion of the mature protein sequence, which encodes for the protein of the present invention, is used to design an antisense RNA oligonucleotide of from 10 to 40 base pairs in length. 5 A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al. *Nucl. Acids Res.*, 6:3073 (1979); Cooney et al., *Science*, 241:456 (1988); and Dervan et al., *Science*, 251: 1360 (1991), thereby 10 preventing transcription and the production of GFR $\alpha$ -4. The antisense RNA oligonucleotide hybridises to the mRNA *in vivo* and blocks translation of an mRNA molecule into the GFR $\alpha$ -4 receptor.

15 Antibodies to the GFR $\alpha$ -4 receptor according to the invention are also provided which may be used in a medicament or in a pharmaceutical composition.

20 Antibodies to the GFR $\alpha$ -4 of the invention may, advantageously, be prepared by techniques which are known in the art. For example, polyclonal antibodies may be prepared by inoculating a host animal, such as a mouse, with the polypeptide according to the invention or an epitope thereof and recovering immune 25 serum. Monoclonal antibodies may be prepared according to known techniques such as described by Kohler R. and Milstein C., *Nature* (1975) 256, 495-497.

30 Antibodies according to the invention may also be used in a method of detecting for the presence of GFR $\alpha$ -4 by reacting the antibody with a sample and identifying any protein bound thereto. A kit may also be provided for performing said method which comprises an antibody according to the invention and means for reacting the 35 antibody with said sample.

40 Advantageously, the antibody according to the invention may also be used as a medicament or in the preparation of a medicament for treating diseases associated with expression of the GFR $\alpha$ -4 of the

invention. The invention also further provides a pharmaceutical composition comprising said antibody together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

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Proteins which interact with the polypeptide of the invention may be identified by investigating protein-protein interactions using the two-hybrid vector system first proposed by Chien et al (1991), Proc. Natl. Acad. Sci. USA 88 : 9578-9582.

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This technique is based on functional reconstitution <sup>15</sup> *in vivo* of a transcription factor which activates a reporter gene. More particularly the technique comprises providing an appropriate host cell with a DNA construct comprising a reporter gene under the control of a promoter regulated by a transcription factor having a DNA binding domain and an activating domain, expressing in the host cell a first hybrid DNA sequence encoding a first fusion of a fragment or all <sup>20</sup> of a nucleic acid sequence according to the invention and either said DNA binding domain or said activating domain of the transcription factor, expressing in the host at least one second hybrid DNA sequence, such as, a library or the like, encoding putative binding <sup>25</sup> proteins to be investigated together with the DNA binding or activating domain of the transcription factor which is not incorporated in the first fusion; detecting any binding of the proteins to be investigated with a protein according to the invention <sup>30</sup> by detecting for the presence of any reporter gene product in the host cell; optionally isolating second hybrid DNA sequences encoding the binding protein. Proteins which bind to the GFR $\alpha$ -4 receptor can be <sup>35</sup> identified using this technique. The proteins identified can also be used to identify compounds which acts as agonists/antagonists of these proteins. The structure of the receptor can also be used to design agonists or antagonists of the receptor. The present invention also comprises an agonist or <sup>40</sup>

5 antagonist of the human GFR $\alpha$ -4 receptor according to the invention which agonist or antagonist advantageously may also be used as a medicament or in a pharmaceutical composition together with a pharmaceutically acceptable carrier diluent or excipient therefor.

10 Agonists or antagonists may be identified by contacting a cell expressing GFR $\alpha$ -4 with a compound to be tested and monitoring the degree of any GFR $\alpha$ -4 mediated functional or biological response, such as for example, by monitoring the level of phosphorylation in said cell or by cytosensor or ligand binding assays in the presence of cRET or similar proteins in the signal transduction pathway. 15 Preferably, the cell may be a host cell or transgenic cell according to the invention as defined herein. Agonists and antagonists of GFR $\alpha$ -4 may also be identified by, for example, contacting a membrane preparation comprising GFR $\alpha$ -4 with the compound to be tested in the presence of cRET or other similar proteins involved in the signal transduction pathway of which GFR $\alpha$ -4 is a component and monitoring the interaction of GFR $\alpha$ -4 with cRET or said similar 20 proteins. Advantageously, any compounds or molecules 25 identified as agonists or antagonists in relation to GFR $\alpha$ -4 may themselves be used in a pharmaceutical composition as defined above or as a medicament.

30 Also provided by the invention are molecules or compounds that act on the signal transduction pathway of which GFR $\alpha$ -4 or a functional equivalent belong. Alternatively, the molecules may interfere with complex formation or interaction of GFR $\alpha$ -4 or its 35 functional equivalent, with cRET or a similar protein in the signal transduction pathway of which GFR $\alpha$ -4 is a component.

40 Furthermore, the present invention relates to a method of producing an antagonist or agonist of GFR $\alpha$ -4

according to the invention comprising the steps of any one of the above described screening methods; and additionally

5 (i) synthesizing the compound obtained or identified in said method or an physiologically acceptable analog or derivative thereof in an amount sufficient to provide said antagonist or agonist in a therapeutically effective amount to a patient; and/or

10 (ii) combining the compound obtained or identified in said method or an analog or derivative thereof with a pharmaceutically acceptable carrier".

15 The compounds isolated by the above methods also serve as lead compounds for the development of analog compounds. The analogs should have a stabilized electronic configuration and molecular conformation that allows key functional groups to be presented to the GFR $\alpha$ -4 receptor in substantially the same way as the lead compound. In particular, the analog compounds have spatial electronic properties which are comparable to the binding region, but can be smaller molecules than the lead compound, frequently having a molecular weight below about 2 kD and preferably below about 1 kD. Identification of analog compounds can be performed through use of techniques such as self-consistent field (SCF) analysis, configuration interaction (CI) analysis, and normal mode dynamics analysis. Computer programs for implementing these techniques are available; e.g., Rein, Computer-Assisted Modeling of Receptor-Ligand Interactions (Alan Liss, New York, 1989). Methods for the preparation of chemical derivatives and analogues are well known to those skilled in the art and are described in, for example, Beilstein, Handbook of Organic Chemistry, Springer edition New York Inc., 175

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Fifth Avenue, New York, N.Y. 10010 U.S.A. and  
Organic Synthesis, Wiley, New York, USA.

Furthermore, said derivatives and analogues can  
be tested for their effects according to methods  
known in the art; see also *supra*. Furthermore,  
5 peptidomimetics and/or computer aided design of  
appropriate derivatives and analogues can be  
used.

10 Compounds identified as agonists or antagonists in  
relation to GFR $\alpha$ -4 or as ligands or compounds which  
interfere with the signal transduction pathway of  
which GFR $\alpha$ -4 is a part, may advantageously be used in  
the preparation of a medicament for treatment of  
15 neurodegenerative diseases, such as, for example,  
Alzheimers disease, Parkinsons disease, Motor Neuron  
Disease, peripheral neuropathy, spinal cord injury,  
familial hirschsprung disease, in addition to various  
carcinomas such as for example in gastrointestinal  
20 cancer and also in treatment of diseases which may be  
associated with GFR $\alpha$ -4 dysfunction. Compounds  
identified as antagonists may, advantageously, be used  
in the preparation of a medicament for the treatment  
of carcinoma or in alleviating pain.

25 The present invention also further comprises a method  
of identifying ligands of GFR $\alpha$ -4 according to the  
invention, which method comprises contacting said  
receptor with either a cell extract or alternatively a  
30 compound to be tested for its potential as a GFR $\alpha$ -4  
ligand, and isolating any molecules bound to GFR $\alpha$ -4.

35 A diagnostic kit is also provided by the present  
invention, which kit, comprises a probe including any  
of, a nucleic acid molecule encoding a GFR $\alpha$ -4 protein  
according to the invention, a molecule capable of  
hybridising thereto under high stringency conditions,  
a fragment of said nucleic acids, an antisense  
molecule according to the invention, together with  
40 means for contacting biological material to be tested

with said nucleic acid probe. A diagnostic kit in accordance with the invention may also comprise an agonist or antagonist in relation to GFR $\alpha$ -4 or an antibody, preferably a monoclonal antibody to GFR $\alpha$ -4. 5 Thus, advantageously, the kit may be used, as appropriate to identify, for example, cells expressing or lacking in said receptor or genetic defects or the like or for determining whether a compound is a agonist or an antagonist of GFR $\alpha$ -4 receptor. Kits for 10 determining whether a compound is an agonist or an antagonist in relation to GFR $\alpha$ -4 may comprise a cell or membrane preparation expressing said receptor according to the present invention, means for 15 contacting said cell with said compound and means for monitoring the level of any GFR $\alpha$ -4 mediated functional or biological response, by for example measuring the level of phosphorylation in said cell or by cytosensor or ligand binding assays in the presence of cRET or 20 similar proteins involved in the signal transduction pathway of which GFR $\alpha$ -4 is a component.

This invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of 25 the invention as described more fully in the claims which follow thereafter. Additionally, throughout this application, various publications are cited. The disclosure of these publications is hereby 30 incorporated by reference into this application to describe more fully the state of the art to which this invention pertains.

35 **Examples**

While the foregoing specification teaches the principles of the present invention, with examples

provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations and/or modifications as come within the scope of the following claims and their equivalents.

5 oligonucleotide synthesis for PCR and DNA sequencing.

10 All oligonucleotide primers used were ordered from Eurogentec (Seraing, Belgium). Insert-specific sequencing primers (15- and 16-mers) and primers for use in PCR reactions were designed manually. DNA was prepared on Qiagen-tip-20 or -100 anion exchange or 15 Qiaquick spin columns (Qiagen GmbH, Düsseldorf, Germany) and recovered from the columns in 30 µl TE-buffer (10 mM Tris.HCl, 1 mM EDTA (sodium salt), pH 8.0). Sequencing reactions were done on both strands using the ABI prism BigDye Terminator Cycle sequencing 20 kit and were run on an Applied Biosystems 377XL sequencer (Perkin Elmer, ABI Division, Foster City, CA, USA). The Sequencher™ software was used for sequence assembly and manual editing (GeneCodes, Ann Arbor, MI, USA).

25 Identification of a cDNA sequence encoding a novel member of the GFRα family

30 Using the human GFRα-1, GFRα-2 or GFRα-3 DNA or protein sequences as the query sequence, BLAST (Basic Local Alignment Search Tool; Altschul et al., 1990) 35 searches were performed on the daily updates of the public EMBL database. A mouse EST (expressed sequence tag) sequence with EMBL accession number AU035938 showed homology to GFRα-1, GFRα-2 and GFRα-3. The smallest sum probabilities (SSP) obtained by the BLAST analyses are summarized in Table 1.

Table 1: BLAST results.

Query sequence	DNA/PROTEIN	SSP
GFRα-1	protein	7.5e-25

		23	JAB-1512
GFR $\alpha$ -2	protein	1.3e-12	
GFR $\alpha$ -3	protein	2.2e-20	
GFR $\alpha$ -1	DNA	6.6e-09	
GFR $\alpha$ -2	DNA	>0.011	
GFR $\alpha$ -3	DNA	0.0096	

AU035938 (sequence 1) is a sequence of 792 bp derived from a mouse brain cDNA library. To obtain consistent homology with other members of the GFR $\alpha$  family upon translation a frame shift has to be introduced near position 165 in the DNA sequence. It is not clear whether this is due to a sequencing error or whether there is another explanation. Using this EST sequence as the query sequence, the BLAST search against the public EMBL database was repeated. One additional clone (acc. no. AA823200; sequence 2) yielded a significant SSP of 1e-18. Upon inspection of this 497 bp clone, which was derived from a mouse mammary gland cDNA library, only the first 61 bp were identical with part of AU035938 (position 353 to 415). The rest of the sequence of AA823200 was different from AU035938, but contained parts of which the translated amino acid sequence showed homology with the other GFR $\alpha$ 's. Therefore it was hypothesized that AU035938 and AA823200 could represent two variant forms of the same receptor, which was called GFR $\alpha$ -4.

#### Cloning of mouse GFR $\alpha$ -4 cDNA

First, we tried to amplify a fragment of the mouse GFR $\alpha$ -4 cDNA on Marathon Ready<sup>TM</sup> cDNAs (Clontech Laboratories, Palo Alto, CA, USA) derived from mouse brain and mouse embryo. Primers were designed using the EST sequences (EMBL acc. no. AU035938 and AA823200) to amplify a 274 bp fragment of mouse GFR $\alpha$ -4. The primers used for the amplification of mouse GFR $\alpha$ -4 are shown in the table below.

Table 2: Primers used for the amplification of mouse GFR $\alpha$ -4 DNA sequences.

Name	Sequence	n
MOUSE-GFR $\alpha$ 4-sp2 (SEQ ID NO:10)	CGCGTTGTCTGCGCGTCTACG	21
MOUSE-GFR $\alpha$ 4-sp3 (SEQ ID NO:11)	CGGCGCGAAGAATGCGAAGC	20
MOUSE-GFR $\alpha$ 4-ap2 (SEQ ID NO:12)	CACCCACGTACCATGGCATGTGC	23

PCR reactions were done using the Taq polymerase system (Boehringer Mannheim, Mannheim, Germany). PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x Taq PCR reaction buffer, 0.25 mM dNTP, 0.5  $\mu$ M of primers MOUSE-GFR $\alpha$ 4-sp2 and MOUSE-GFR $\alpha$ 4-ap2, 1  $\mu$ l of Taq polymerase and 2  $\mu$ l of mouse embryo or mouse brain Marathon Ready<sup>TM</sup> cDNA. Samples were heated to 95°C for 5 min and cycling was done for 30 s at 94°C, 1 min at 60°C and 45 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. A semi-nested PCR was then performed on 1  $\mu$ l of the primary PCR reaction with primers MOUSE-GFR $\alpha$ 4-sp3 and MOUSE-GFR $\alpha$ 4-ap2. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x Taq PCR reaction buffer, 0.25 mM dNTP, 0.5  $\mu$ M of primers MOUSE-GFR $\alpha$ 4-sp3 and MOUSE-GFR $\alpha$ 4-ap2, 1  $\mu$ l of Taq polymerase and 1  $\mu$ l of primary PCR product. Samples were heated to 95°C for 5 min and cycling was done for 30 s at 94°C, 1 min at 60°C and 45 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). A PCR fragment of the expected size (270 bp) was excised from the gel and purified with the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The PCR fragments were sequenced to confirm their identity. The obtained sequence corresponded to the EST database sequences. In order to determine the upstream and downstream coding sequences of mouse GFR $\alpha$ -4, 5' and 3' RACE experiments were performed. Since these experiments did not work as expected and since, at some points,

frame shifts had to be introduced in the mouse GFR $\alpha$ -4 sequence to yield consistent homology with other GFR $\alpha$ 's after translation, we decided to shift to the cloning of the rat homologue of mouse GFR $\alpha$ -4.

5

Identification and cloning of rat GFR $\alpha$ -4 cDNA sequences

The cDNA sequences with accession number AU035938 and AA823200 described above were used as the query sequence in BLAST searches on the proprietary LifeSeq and ZooSeq databases (Incyte Pharmaceuticals, Palo Alto, CA, USA). Two rat clones with high homology to the mouse GFR $\alpha$ -4 sequences were identified: number 15 701290919H1 (270 bp; hit with AU035938 (SSP = 1.1e-32) and with AA823200 (SSP = 1.3e-21)) and number 701291473H1 (250 bp; hit only with AA823200 (SSP = 4.3e-42)). From comparing the translated protein sequences derived from clones 701291473H1 and 20 701290919H1 to the known GFR $\alpha$  protein sequences, it could be deduced that sequence 701290919H1 was probably localised 5' to sequence 701291473H1 and that these sequences were almost adjacent to each other in the full GFR $\alpha$ -4 cDNA sequence. Therefore, two forward 25 primers (RAT-GFR $\alpha$ 4-sp1 and RAT-GFR $\alpha$ 4-sp2) were designed in the 5' region of sequence 701290919H1 and two reverse primers (RAT-GFR $\alpha$ 4-ap1 and RAT-GFR $\alpha$ 4-ap2) in the 3' region of sequence 701291473H1. All primer sequences used in PCR experiments are summarized in 30 Table 3.

**Table 3:** Primers used for the amplification of rat GFR $\alpha$ -4 sequences. The RACE-ap1 and RACE-ap2 primers are included in the Marathon Ready<sup>TM</sup> cDNA kit.

5

Name	Sequence	n
RAT-GFR $\alpha$ 4-sp1 (SEQ IDNO:13)	GTGGTCACCCCCAACTACCTGG	22
RAT-GFR $\alpha$ 4-sp2 (SEQ IDNO:14)	GCCTTCCGCAAGCTTTACAAGG	24
RAT-GFR $\alpha$ 4-sp3 (SEQ IDNO:15)	GCTCTTCTGCGGATGCGAAGGC	22
RAT-GFR $\alpha$ 4-sp4 (SEQ IDNO:16)	AGCTGCCGGTTTACTGATGCTAC	24
RAT-GFR $\alpha$ 4-sp5 (SEQ IDNO:17)	GATGCTACTCTCCAAGGTCAGGC	24
RAT-GFR $\alpha$ 4-sp6 (SEQ IDNO:18)	CTGGTAAGCTTAAGGCAGAGGAGACC	27
RAT-GFR $\alpha$ 4-ap1 (SEQ IDNO:19)	CATGGCAGTCAGCTGTGTTGTCC	23
RAT-GFR $\alpha$ 4-ap2 (SEQ IDNO:20)	CAGCTGTGTTGTCCATGGTCACC	24
RAT-GFR $\alpha$ 4-ap3 (SEQ IDNO:21)	TGGTTGCGAGCTGTCAAAGGCTTGTATGGC	30
RAT-GFR $\alpha$ 4-ap4 (SEQ IDNO:22)	GGGGTTCCCTTGTAAGGCTTGCAGGAAGGC	30
RAT-GFR $\alpha$ 4-ap5 (SEQ IDNO:23)	GGTCCAAGGGCTTCAGGCAGGAAGG	25
RAT-GFR $\alpha$ 4-ap6 (SEQ IDNO:24)	GCCTTCGCATCCGCAGAAGAGC	22
RAT-GFR $\alpha$ 4-ap7 (SEQ IDNO:25)	CCAGGTAGTTGGGGTGACCAAG	23
RAT-GFR $\alpha$ 4- ap7b (SEQ IDNO:26)	CCCAGGCATTGCGCCACGTA	20
RAT-GFR $\alpha$ 4-ap8 (SEQ IDNO:27)	CATTGCGCCACGTACTCGGAGC	22
RAT-GFR $\alpha$ 4-ap9 (SEQ IDNO:28)	GACCTGAGGGCAAGGGAGTTCA	23
RAT-GFR $\alpha$ 4- ap10 (SEQ IDNO:29)	GCAAGGGAGTTTCAGTTCACTGAGC	25
RACE-ap1 (SEQID NO:30)	CCATCCTAATACGACTCACTATAGGGC	27

RACE-ap2 (SEQ IDNO:31)	ACTCACTATAGGGCTCGAGCGGC	23
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A PCR was then performed using primers RAT-GFR $\alpha$ 4-sp1 and RAT-GFR $\alpha$ 4-ap1 on rat brain Quickclone cDNA (Clontech Laboratories, Palo Alto, CA, USA) to confirm the presence of rat GFR $\alpha$ -4 in brain-derived cDNA. Since the DNA sequence coding for the rat GFR $\alpha$ -4 sequence has a high G+C content in this region, PCR reactions were done using the Advantage-GC PCR kit (Clontech). PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT $\ddot{a}$ , 200 nM of primers RAT-GFR $\alpha$ 4-sp1 and RAT-GFR $\alpha$ 4-ap1, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 1  $\mu$ l of rat brain Quickclone cDNA. Samples were heated to 95°C for 1 min and cycling was done for 1 min at 95°C, 1 min at 56°C and 1 min at 72°C for 30 cycles, with a final step of 7 min at 72°C. A nested PCR was then performed on 1  $\mu$ l of the primary PCR reaction with primers RAT-GFR $\alpha$ 4-sp2 and RAT-GFR $\alpha$ 4-ap2. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT $\ddot{a}$ , 200 nM of primers RAT-GFR $\alpha$ 4-sp2 and RAT-GFR $\alpha$ 4-ap2, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 1  $\mu$ l of primary PCR product. Samples were heated to 95°C for 1 min and cycling was done for 30 s at 95°C, 1 min at 56°C and 1 min at 72°C for 25 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). Two PCR fragments of approximately 1100 and 200 bp, respectively, were excised from the gel and purified with the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The PCR fragments were sequenced to confirm their identity. The smallest fragment yielded a sequence of 211 bp corresponding to the joined sequences 701290919H1 and 701291473H1. The larger fragment

yielded a sequence of 1049 bp of which 18 bp at the 5' end, 59 bp at the 3' end and an internal stretch of 92 bp corresponded to the sequence of the 211 bp fragment, but which had additional sequence stretches in between. This fragment represented a variant of rat GFR $\alpha$ -4.

Both clones 701291919H1 and 701291473H1 were obtained from Incyte Pharmaceuticals and the inserts completely sequenced. The sequences are included in this application (sequence 3 = 701290919H1 and sequence 4 = 701291473H1). Both clones were derived from the same 7-day old rat brain cortex cDNA library. Both clones differ in their 5' ends (first 134 bp in 701291473H1 and first 227 bp in 701290919H1) but are identical thereafter. Both contain part of the GFR $\alpha$ -4 coding sequence up to a stop codon (position 184-186 in 701291473H1 and 277-279 in 701290919H1). A 3' untranslated region of 549 bp followed by a poly(A)-tail is then present in both clones. We hypothesized that both clones are different variants of the rat GFR $\alpha$ -4 gene. Primers (RAT-GFR $\alpha$ 4-ap3 and RAT-GFR $\alpha$ 4-ap4) were designed on a part of the sequence common to both variants to perform 5' RACE experiments in order to determine the 5' end of the rat GFR $\alpha$ -4 cDNA.

First, a 5' RACE PCR was performed on rat brain Marathon Ready<sup>TM</sup> cDNA (Clontech). PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M or 1.5 M GC-MELT $\alpha$ , 200 nM of primers RAT-GFR $\alpha$ 4-ap3 and RACE-ap1, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 5  $\mu$ l of rat brain Marathon Ready<sup>TM</sup> cDNA. Samples were heated to 95°C for 30 s and cycling was done for 30 s at 95°C, 4 min at 72°C for 5 cycles, then 30 s at 95°C, 4 min at 70°C for 5 cycles, then 30 s at 95°C, 4 min at 68°C for 25 cycles, with a final step of 7 min at 68°C. A nested PCR was then performed on 1  $\mu$ l of the primary PCR reaction with primers RAT-GFR $\alpha$ 4-ap4 and

RACE-ap2. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M or 1.5 M GC-MELT $\ddot{\alpha}$ , 200 nM of primers RAT-GFR $\alpha$ 4-ap4 and RACE-ap2, 1  $\mu$ l of Advantage 5 KlenTaq polymerase mix and 1  $\mu$ l of primary PCR product. Cycling was done using exactly the same parameters as for the primary PCR. PCR products were analysed on a 1% (w/v) agarose gel in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA (sodium salt), pH 8.3). 10 A fragment of approximately 350 bp was excised from the gel and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit according to manufacturer's instructions (Invitrogen BV, Leek, The Netherlands). One of the resulting clones yielded an 15 insert sequence of 387 bp which extended the rat GFR $\alpha$ -4 sequence in the 5' direction. Upon translation, this additional cDNA sequence yielded a protein sequence without any internal stop codons and with substantial homology to the other known GFR $\alpha$  sequences. Since no 20 putative ATG start codon could be detected within this additional sequence, novel primers (RAT-GFR $\alpha$ 4-ap5 and RAT-GFR $\alpha$ 4-ap6) were designed at the 5' end of this sequence to perform additional 5' RACE experiments. First, a 5' RACE PCR was performed on rat heart 25 Marathon Ready $^{\text{TM}}$  cDNA (Clontech). PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELT $\ddot{\alpha}$ , 200 nM of primers RAT-GFR $\alpha$ 4-ap5 and RACE-ap1, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 5  $\mu$ l of rat heart 30 Marathon Ready $^{\text{TM}}$  cDNA. Samples were heated to 95°C for 30 s and cycling was done for 30 s at 95°C, 4 min at 72°C for 5 cycles, then 30 s at 95°C, 4 min at 70°C for 5 cycles, then 30 s at 95°C, 4 min at 68°C for 25 cycles, with a final step of 7 min at 68°C. A nested 35 PCR was then performed on 1  $\mu$ l of the primary PCR reaction with primers RAT-GFR $\alpha$ 4-ap6 and RACE-ap2. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM

dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-ap6 and RACE-ap2, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 1  $\mu$ l of primary PCR product. Cycling was done using exactly the same parameters as for the primary PCR. PCR products were analysed on a 1% agarose gel. A fragment of approximately 200 bp was excised from the gel and cloned into the plasmid vector pCR2.1-TOPO using the TOPO TA cloning kit as described above. Sequencing of two resulting clones extended the rat GFR $\alpha$ -4 sequence with another 128 bp in the 5' direction. Based on this sequence, another primer set (RAT-GFR $\alpha$ 4-ap7 and RAT-GFR $\alpha$ 4-ap8) was designed to perform additional 5' RACE experiments. RACE PCR was performed on rat brain, heart and kidney Marathon Ready<sup>TM</sup> cDNA. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-ap7b and RACE-ap1, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 5  $\mu$ l of rat heart, brain or kidney Marathon Ready<sup>TM</sup> cDNA. Samples were heated to 95°C for 30 s and cycling was done for 30 s at 95°C, 4 min at 72°C for 5 cycles, then 30 s at 95°C, 4 min at 70°C for 5 cycles, then 30 s at 95°C, 4 min at 68°C for 25 cycles, with a final step of 7 min at 68°C. A nested PCR was then performed on 1  $\mu$ l of the primary PCR reaction with primers RAT-GFR $\alpha$ 4-ap8 and RACE-ap2. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-ap8 and RACE-ap2, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 1  $\mu$ l of primary PCR product. Cycling was done using exactly the same parameters as for the primary PCR. PCR products were analysed on a 1% agarose gel. Several fragments ranging in size from approximately 200 bp to 1200 bp were visible on the gel and were excised and cloned in vector pCR2.1-TOPO using TOPO-TA cloning. The inserts of several clones were sequenced. From these clones, the sequence of rat GFR $\alpha$ -4 could be

extended in the 5' direction. Two different sequences were identified. One sequence extended the ratGFR $\alpha$ -4 sequence with 215 bp in the 5' direction and included an in-frame start codon preceded by an in-frame upstream stop codon. The resulting predicted protein sequence (52 additional amino acid residues) includes a predicted signal peptide of 29 amino acid residues (as determined by the SPScan program included in the Wisconsin package version 10.0, Genetics Computer Group (GCG), Madison, Wisconsin, USA; score 7.0, probability 1.171e-02). The other sequence determined by these 5' RACE experiments extended the ratGFR $\alpha$ -4 sequence with 552 bp in the 5' direction and also included an in-frame start codon preceded by an in-frame upstream stop codon. The most 3' 79 base pairs of this novel sequence were identical to the 3' 79 base pairs of the 215 bp sequence, but the rest of the sequence was different. The resulting predicted protein sequence (113 additional amino acid residues), however, did not have a predicted signal peptide sequence at the NH<sub>2</sub>-terminus (SPScan, GCG package). The different partial cDNA sequences resulting from the subsequent 5' RACE experiments together with the sequences from the Incyte database were compared and merged into several possible rat GFR $\alpha$ -4 variants. In order to identify which of the identified variants are real, primers were designed 5' of the translation start codon (primers RAT-GFR $\alpha$ 4-sp4 and RAT-GFR $\alpha$ 4-sp5 for the "long" 5' variant resulting from the 552 bp RACE fragment and RAT-GFR $\alpha$ 4-sp6 for the "short" 5' variant resulting from the 215 bp RACE fragment) and 3' of the translation stop codon (RAT-GFR $\alpha$ 4-ap9 and RAT-GFR $\alpha$ 4-ap10). These primers were then used to amplify the full GFR $\alpha$ -4 coding sequences using cDNA derived from different rat tissues. First, sequences coding for the "long" 5' variant were amplified by PCR. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR

reaction buffer, 0.2 mM dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-sp4 and RAT-GFR $\alpha$ 4-ap9, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 5  $\mu$ l of rat heart, brain or kidney Marathon Ready<sup>TM</sup> cDNA. Samples were heated to 95°C for 1 min and cycling was done for 45 s at 95°C, 1 min at 57°C and 1 min at 72°C for 35 cycles, with a final step of 7 min at 72°C. A nested PCR was then performed on the primary PCR reaction with primers RAT-GFR $\alpha$ 4-sp5 and RAT-GFR $\alpha$ 4-ap10. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-sp5 and RAT-GFR $\alpha$ 4-ap10, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 1  $\mu$ l of primary PCR product.

Cycling was done using exactly the same parameters as for the primary PCR, except that 30 PCR cycles were done instead of 35. PCR products were analysed on a 1% agarose gel. Several fragments ranging in size from approximately 1000 to 1250 bp were excised from gel and cloned in vector pCR2.1-TOPO using TOPO-TA cloning. The inserts of several clones were sequenced. Next, sequences coding for the "short" 5' variant were amplified by PCR. PCR reactions were performed in a total volume of 50  $\mu$ l, containing 1x GC cDNA PCR reaction buffer, 0.2 mM dNTP, 1 M GC-MELTä, 200 nM of primers RAT-GFR $\alpha$ 4-sp6 and RAT-GFR $\alpha$ 4-ap9, 1  $\mu$ l of Advantage KlenTaq polymerase mix and 5  $\mu$ l of rat heart Marathon Ready<sup>TM</sup> cDNA. Samples were heated to 95°C for 5 min and cycling was done for 30 s at 94°C, 1 min at 57°C and 2 min 30 s at 72°C for 35 cycles, with a final step of 7 min at 72°C. PCR products were analysed on a 1% agarose gel. Several fragments ranging in size from approximately 1500 to 2200 bp were excised from gel and cloned in vector pCR2.1-TOPO using TOPO-TA cloning. The inserts of several clones were sequenced. Analysis of all the obtained sequences (16 resulting clones were completely sequenced) allowed the rat GFR $\alpha$ -4 DNA sequence to be divided into

6 sequence stretches common to all identified variants, with 5 intervening sequence stretches present or absent depending on the variant. All 5 intervening sequences contain 5' and 3' splice site consensus sites (GT at the 5' end and AG at the 3' end of the intron sequence) (Senapathy et al., 1990) (see table 4 below) and could thus potentially represent unspliced introns.

In order to strengthen the hypothesis that the identified variants could result from the conservation of unspliced introns in certain mRNA transcripts, the rat GFR $\alpha$ -4 sequence was compared to the genomic sequence of human GFR $\alpha$ -1 (Angrist et al., 1998). From this analysis, it was apparent that the GFR $\alpha$ -4 sequences common to all transcripts coincided with exons in GFR $\alpha$ -1 (see table 4 below). The intervening sequences absent in some transcripts coincided with intron sequences in human GFR $\alpha$ -1. Therefore, we considered all intervening sequences as unspliced introns. The intron present between exon 5 and exon 6 can be spliced out in two different ways and results in the presence of two different splice variants of rat GFR $\alpha$ -4, which we have called variant A and variant B.

Sequence 5 shows the consensus sequence for rat GFR $\alpha$ -4 including the intron sequences (intron 1: bp 125 to 684; intron 2: bp 1040 to 1088; intron 3: bp 1199 to 1278; intron 4: bp 1414 to 2154; intron 5A: bp 2247 to 2385 and intron 5B: bp 2231 to 2314). A polymorphism was detected at position 2244 in sequence 5, with T found in 50% of the sequenced clones and C in the other 50%. This polymorphism leads to an amino acid change in the protein (variant A) from W to R, in the hydrophobic region involved in GPI-anchoring. Figure 1 schematically shows the structure of the rat GFR $\alpha$ -4 gene together with the derived cDNA for splice

variants A and B after splicing out of the intron sequences and the translated protein sequences of variants A and B with their characteristics.

5 Table 4 shows the DNA sequence at the intron-exon boundaries together with the sizes of identified introns and exons. The right column shows the sizes of the corresponding exons in the genomic sequence of human GFR $\alpha$ -1 (from Angrist et al., 1998).

10 **Table 4:** Intron-exon structure of rat GFR $\alpha$ -4.

Exon	Size (bp)	Intron size (bp)	Splice acceptor	Splice donor	Corresponding GFR $\alpha$ -1 exon size (bp)
1	>124	560	---	GAGgttaaggaggt	---
2	355	49	ccctcaccagGGT	CCGgtgcgtgcgg	337
3	110	80	gcgcgcgcagGCC	TAGgtacgcgtggg	110
4	135	741	gtccctgcagGCA	TGGgtgaggggggc	135
5	92	139 (varA) 84 (varB)	cactccatagATG	CGGgttaggtatgg TGGgtgctgtttc	182
6	>137	---	ttgtcccaagGTG cccttctcagGCA	-	753

15 The consensus sequence obtained by removing introns 1 to 4 and intron 5A (sequence 6; variant A) translates into a protein of 273 amino acid residues with a calculated molecular mass of 29.7 kDa and an isoelectric point of 8.92 (sequence 8). The consensus sequence obtained by removing introns 1 to 4 and intron 5B (sequence 7; variant B) translates into a protein of 258 amino acid residues with a calculated molecular mass of 28.0 kDa and an isoelectric point of 8.91 (sequence 9). Figure 2 shows the alignment of variants A and B of rat GFR $\alpha$ -4. The protein sequences are both similar to the known GFR $\alpha$  sequences and only differ from each other in a small amino acid stretch 20 at the carboxy-terminus. These two sequences probably represent biologically active GFR $\alpha$ -4 variants. Since all the other variants sequenced contain one or more 25 intron sequences, they are probably intermediates of RNA processing. It is not clear why all these 30

intermediates are present in cDNA derived from purified mRNA and why it is so difficult to amplify a cDNA sequence derived from a completely spliced mRNA transcript. GFR $\alpha$ -1 to -4 are characterized by a COOH-terminal sequence typical of a glycosyl-phosphatidyl inositol (GPI)-anchored protein, consisting of a hydrophobic region of 17-31 amino acid residues preceded by a hydrophilic sequence containing a stretch of three, small amino acids such as Asp, Cys, Ala, Ser, Gly or Asn (Gerber et al., 1992). The rat GFR $\alpha$ -4 variant A protein sequence has a hydrophobic carboxy-terminus of 21 amino acid residues (position 253 to 273) preceded by two possible GPI cleavage sites (DSS at position 234 to 236 or NAG at position 250-252). Variant B has a shorter hydrophilic carboxy-terminus, implying that no GPI-anchoring is possible for this variant. This could mean that variant B is a soluble form of the rat GFR $\alpha$ -4 receptor. A predicted signal peptide of 29 amino acids is present in both variants (as determined by the SPScan program included in the GCG package; score 7.0, probability 1.171e-02). In addition, one possible site for N-linked glycosylation (NVS at position 192 to 194 in the protein) is present.

Recently, a model has been proposed for the domain structure of GFR $\alpha$ 's based on the comparison of the sequences of mouse GFR $\alpha$ -1 to -3 and chicken GFR $\alpha$ -4 (Airaksinen et al., 1999). The model includes three conserved cysteine-rich domains joined together by less conserved adaptor sequences. The molecules are anchored to the membrane by a GPI-anchor. Rat GFR $\alpha$ -4 conforms partly to this model, since it also contains the second and third cysteine-rich region and a possible GPI-anchor (at least for variant A). However, it differs significantly from the other GFR $\alpha$ 's in that the first cysteine-rich region is absent.

Figure 3 shows the alignment of rat GFR $\alpha$ -4 variants A

and B with rat GFR $\alpha$ -1 (EMBL acc. no. U59486), rat GFR $\alpha$ -2 (EMBL acc. no. AF003825), mouse GFR $\alpha$ -3 (EMBL acc. no. AB008833) and chicken GFR $\alpha$ -4 (EMBL acc. no. AF045162). The alignment was done using the ClustalW 5 alignment program (EMBL, Heidelberg, Germany). The percentage identity and percentage similarity between members of the GFR $\alpha$  family were calculated by pairwise comparison of the sequences using the GeneDoc software tool (version 2.5.000) and the results are presented 10 in Table 5 below.

Table 5: % identity and % similarity (between 15 brackets) between members of the GFR $\alpha$  family. Accession numbers of the sequences used in the analysis are mentioned in the text.

	rGFR $\alpha$ -1	rGFR $\alpha$ -2	mGFR $\alpha$ -3	cGFR $\alpha$ -4	rGFR $\alpha$ -4 (A)	rGFR $\alpha$ -4 (B)
rGFR $\alpha$ -1	100	43 (60)	15 (23)	38 (55)	20 (29)	20 (28)
rGFR $\alpha$ -2		100	18 (28)	40 (56)	21 (32)	21 (31)
mGFR $\alpha$ -3			100	16 (25)	22 (30)	20 (29)
cGFR $\alpha$ -4				100	27 (37)	26 (35)
rGFR $\alpha$ -4 (A)					100	92 (92)
rGFR $\alpha$ -4 (B)						100

Four members of the GDNF family of neurotrophic 20 factors have been identified so far (GDNF, NTN, PSP, EVN/ARTN). All four signal through binding to a specific GPI-linked GFR $\alpha$  receptor (GFR $\alpha$ -1 for GDNF, GFR $\alpha$ -2 for NTN, GFR $\alpha$ -3 for EVN/ARTN and (chicken) GFR $\alpha$ -4 for PSP) in combination with a common 25 transmembrane tyrosine kinase, CRET. GFR $\alpha$ -4, the coreceptor for PSP, has been identified in chicken only and no mammalian counterpart has been found yet.

The similarity between the rat GFR $\alpha$ -4 described in the 30 present application and the chicken GFR $\alpha$ -4 is 37% (27% identity) suggesting that rat GFR $\alpha$ -4 is a novel member of the GFR $\alpha$  family. GFR $\alpha$ -4 could be the mammalian

persephin receptor or, alternatively, could be the receptor for an unidentified GDNF family member.

Specific binding of persephin to GFR $\alpha$ -4.

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Constructs for the expression of soluble GFR $\alpha$ -IgG-Fc fusion proteins were made as follows. cDNA regions of human GFR $\alpha$ -1, GFR $\alpha$ -2 and GFR $\alpha$ -3, chicken GFR $\alpha$ -4 and rat GFR $\alpha$ -4 variant A (coding for amino acid residues 27 to 427, 20 to 431, 28 to 371, 20 to 399 and 29 to 252, respectively), excluding the sequences coding for the signal peptide and for the COOH-terminal hydrophobic region involved in GPI-anchoring, were cloned in-frame in the expression vector Signal pIg plus (R&D Systems Europe Ltd, Abingdon, UK). The inserts of all constructs were confirmed by complete DNA sequence analysis. The resulting proteins expressed from these constructs contain a 17 amino acid residue NH<sub>2</sub>-terminal CD33 signal peptide, the respective GFR $\alpha$  protein region and a 243 amino acid residue COOH-terminal human IgG<sub>1</sub>-Fc fusion domain. Fusion proteins were expressed in CHO cells and purified as described. Chinese hamster ovary (CHO) cells were routinely cultured in DMEM/F12 medium supplemented with 10 % heat inactivated fetal calf serum. Cells were transfected with GFR $\alpha$ -IgGFC fusion constructs using an optimized Lipofectamine Plus method. For this, a total amount of 6.5  $\mu$ g DNA was incubated with 17.5  $\mu$ l PLUS reagent in 750  $\mu$ l serum free medium for 15 min at room temperature. Lipofectamine was diluted 50-fold into serum free culture medium, 750  $\mu$ l of this mixture was added to the DNA solution. Following a 15 min incubation at room temperature, 3.5 ml serum free medium was added, and the mixture was brought onto the cells (in a 100 mm petridish). The cells were incubated for 3h at 37°C in 5 % CO<sub>2</sub>, after which 5 ml of culture medium, containing 20 % heat inactivated fetal calf serum, was

added. 24 h later, the medium was changed into regular culture medium. Transfection efficiencies using these optimized conditions were typically 50-60%. For permanent transfections the selection medium contained either 800  $\mu$ g G418 or 800  $\mu$ g G418 and 800  $\mu$ g hygromycin. Antibiotic resistant clones were expanded and assayed for expression using specific antibodies. GFR $\alpha$ -IgGFC fusion proteins were purified from the medium of permanently or transiently transfected CHO cells by protein A chromatography. Bound protein was eluted with 0.1 M Na-citrate, pH 3.0 and collected into 1 M Tris buffer, pH 8.4 (dilution ratio 1:6). Protein concentration was estimated by absorbance at 280 nm using an extinction coefficient of 1.5. Surface plasmon resonance (SPR) experiments were performed at 25°C using a BIACORE 3000 instrument (Biacore AB, Uppsala, Sweden). Sensor chip CM5, the amine coupling kit and buffers used were also obtained from Biacore AB. Recombinant PSP, NTN, EVN/ART and GDNF were used as immobilised ligands. Recombinant human GDNF was obtained from R&D Systems Europe Ltd. (Abingdon, UK). NH<sub>2</sub>-terminally 6His-tagged recombinant human NTN, rat PSP and human EVN/ART were produced in *E. coli* as described previously (Creedon et al., 1997). The carboxylated matrix of a CM5 sensor chip was first activated with a 1:1 mixture of 400 mM N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide and 100 mM N-hydroxy-succinimide for 10 min. Recombinant neurotrophic factors were applied onto the activated surface in 10 mM sodium acetate buffer, pH 4.5 at a flow rate of 5  $\mu$ l/min. Unreacted carboxyl groups were blocked with 1 M ethanalamine-HCl. For binding experiments, soluble GFR $\alpha$ -IgGFC fusion proteins were superfused using the kinject program at 30  $\mu$ l/min. Concentrations of GFR $\alpha$ -IgGFC used in kinetic experiments were between 1 and 100 nM in Hepes buffered saline (150 mM NaCl, 3.5 mM EDTA sodium salt, 0.005% polysorbate 20, 10 mM Hepes, pH 7.4). The

association of the GFR $\alpha$  receptors to the immobilised ligands was monitored for 3 min and the dissociation for 1 min, followed by regeneration with 10 mM glycine buffer. Dissociation was initiated by superfusion with Hepes buffered saline. To improve the quality of sensor data, double referencing was used (Myszka, 1999). Data were analyzed using a global analysis with the BIACORE evaluation software (version 3.0.1). Global analysis calculates the association rate ( $k_a$ ) and dissociation rate ( $k_d$ ) simultaneously and the apparent equilibrium dissociation constant ( $K_D$ ) is then calculated as  $k_d/k_a$ . A simple 1:1 Langmuir model was used to fit the data. Specific binding to PSP could be detected with both rat and chicken GFR $\alpha$ -4-IgGFc fusion proteins. The observed binding of GFR $\alpha$ -4-IgGFc was specific as there was no binding to GDNF, NTN or EVN/ART. Control experiments confirmed binding of GFR-1 to GDNF, of GFR $\alpha$ -2 to NTN and of GFR $\alpha$ -3 to EVN/ART. From the binding curves obtained using three determinations at differing concentrations of rat and chicken GFR $\alpha$ -4-IgGFc, the binding constants  $k_a$  (association rate) and  $k_d$  (dissociation rate) were derived (Table 6).

Table 6: Persephin binding to chicken GFR $\alpha$ -4 and rat GFR $\alpha$ -4.

Binding constants for chicken GFR $\alpha$ -4-IgGFc and rat GFR $\alpha$ -4-IgGFc binding to immobilised persephin as determined by SPR. The mean association rate ( $k_a$ ), dissociation rate ( $k_d$ ) and apparent equilibrium dissociation constant ( $K_D$ )  $\pm$  standard errors were derived from the binding curves obtained using 3 determinations at differing concentrations of the respective soluble receptors.

	$k_a$ (1/Ms)	$k_d$ (1/s)	$K_D$ (M)
chicken	$2.3 \pm 2.6$ $\times 10^4$	$8.8 \pm 5.1 \times 10^{-4}$	$5.4 \pm 2.9 \times 10^{-9}$
GFR $\alpha$ -4			
rat GFR $\alpha$ -4	$2.7 \pm 1.6$ $\times 10^4$	$1.1 \pm 0.2 \times 10^{-3}$	$5.9 \pm 2.8 \times 10^{-9}$

5      Although apparent  $K_D$  values were very similar for both fusion proteins,  $R_{MAX}$  values were significantly different. Binding levels of ~1000 relative units (RU) were routinely obtained with chicken GFR $\alpha$ -4-IgGFC, whereas binding levels of rat GFR $\alpha$ -4-IgGFC were approximately 20 times lower, around 50-60 RU. This could be due to differences in the concentration of active chicken GFR-4-IgGFC and rat GFR $\alpha$ -4-IgGFC fusion protein. The calculated equilibrium dissociation constant  $K_D$  of  $5.9 \pm 2.8$  nM (n=3) suggests that rat GFR $\alpha$ -4 is a receptor specific for persephin.

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#### Northern blot analysis.

20      Northern blots containing 2  $\mu$ g of poly(A)-rich RNA derived from different rodent tissues (mouse MTN<sup>TM</sup> blot, mouse embryo MTN<sup>TM</sup> blot and rat MTN<sup>TM</sup> blot; Clontech Laboratories) were hybridized according to the manufacturer's instructions with a  $\alpha$ -[<sup>32</sup>P]-dCTP random-priming labeled (HighPrime kit, Roche Diagnostics) 948 bp fragment derived from the rat GFR $\alpha$ -4 coding sequence (as in sequence ID No. 6). Stringency washes were performed in 0.1x SSC / 0.1 % SDS at 50°C (the two mouse blots) or 55°C (the rat blot). The results are shown in Figure 4. In rat, a very weak signal could be detected around 2.3 kb in heart, brain, liver and testis. An even weaker second transcript was present around 1.4 kb in the same tissues. In mouse, a 1.35 kb transcript was most

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intense in brain and testis, with a much weaker signal present around 2 kb. Very low mRNA expression of the 1.35 kb transcript was also present in 15-day and 17-day mouse embryo. The size of the smaller transcript 5 is in agreement with the predicted size of the GFR $\alpha$ -4 coding sequence ( $\pm$  880 bp + the 3' untranslated region of 570 bp).

10 Chromosomal localization of rat, mouse and human GFR $\alpha$ -4.

A 0.95 kb rat GFR $\alpha$ -4 cDNA fragment (containing the full rat GFR $\alpha$ -4 coding sequence of variant A) and a 2.3 kb fragment corresponding to the rat GFR $\alpha$ -4 15 genomic sequence (see sequence ID No. 5) were used as probes in fluorescent *in situ* hybridization (FISH) analysis on rat chromosomes. These probes were partially overlapping. A mixture of the two probes (1  $\mu$ g total DNA) was labeled with digoxigenin-11-dUTP 20 (Roche Diagnostics, Mannheim, Germany) by nick translation (Life Technologies) producing a final probe fragment size of 200-400 bp. The labeled probe was mixed with hybridization buffer (50% formamide, 2x SSC, 10% dextran sulfate). After denaturation, the 25 mixture was placed on metaphase rat chromosome slides (Islam and Levan, 1987, Helou et al., 1998) denatured at 72°C for 2 min in 70% formamide, 2x SSC. After hybridization for 48 h at 37°C, preparations were washed for 15 min in 50 % formamide, 2x SSC. Detection 30 of labeled chromosomes was done by standard FITC anti-digoxigenin. Chromosome spreads were counterstained with 4', 6-diamidino-2-phenylindole (DAPI). Results were derived from micrographs of 100 different cells. Due to the small size of the probes there was considerable background. However, most studied cells 35 showed label at position RNO3q36 (Figure 5). About 35 % of the metaphase studies showed "double spot" label at both homologues of RNO3, whereas about 50 % had

double spots on only one of the homologues or "single spot" label on both homologues. No other chromosomal site showed label in several cells. Based on comparative mapping, the corresponding mouse locus would be expected to be located at MMU2 (band F), whereas a possible human location for the gene would be HSA2, HSA15 or HSA20. In agreement with this, the genomic mouse and human GFR $\alpha$ -4 sequences identified in the EMBL database (Accession No. AF155960 for mouse and AC017113 for human) are derived from mouse chromosome 2 (BAC clone 389B9) and from human chromosome 2 (EMBL accession number AC013324; BAC388\_K\_24map2), respectively.

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List of abbreviations

ARTN	artemin
BLAST	basic local alignment search tool
5 bp	base pairs
CDNA	complementary DNA
CNS	central nervous system
EST	expressed sequence tag
EVN	enovin
10 GDNF	glial cell-line derived neurotrophic factor
GFR $\alpha$	GDNF family receptor $\alpha$
GPI	glycosyl phosphatidyl inositol
NTN	neurturin
15 PCR	polymerase chain reaction
PNS	peripheral nervous system
PSP	persephin
SSP	smallest sum probability
TGF- $\beta$	transforming growth factor $\beta$

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